Cloning and expression analysis of a UBX domain-containing protein gene *LmUBX*2 from the migratory locust, *Locusta migratoria manilensis* (Orthoptera: Locustidae)

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Abstract: 【Aim】 Ubiquitin regulatory X (UBX) domain-containing proteins function as cofactors of p97/CDC48 which is involved in multiple ubiquitin-related processes including protein degradation through the ubiquitin-proteasome system and homotypic membrane fusion. Our study aims to clone the UBX domain-containing protein gene from *Locusta migratoria manilensis*, and to analyze its expression patterns in different tissues and at different developmental stages, so as to provide the foundation for further function research. 【Methods】 A transcriptome database for *L. migratoria manilensis* was mined through bioinformatic analysis. Gene expression levels were analyzed in different tissues of *L. migratoria manilensis* adults and at different developmental stages using real-time PCR. 【Results】 A UBX domain-containing protein gene was identified, and named *LmUBX*2. The open reading frame (ORF) encodes a protein of 339 amino acids with a predicted molecular weight of 37.8 kDa and a theoretical pI of 6.03. BLAST analysis showed that it shares 37% -64% amino acid sequence identities to other UBX domain-containing proteins. LmUBX2 possesses a conserved N-terminal UBA domain and a C-terminal UBX domain. Sequence comparison and phylogenetic analysis revealed that LmUBX2 is a member of the SAKS1 subfamily. Gene expression analysis results showed that *LmUBX*2 was expressed during the whole life cycle, and highly expressed in ovary and testis. 【Conclusion】 These findings suggest that *LmUBX*2 might be involved in multiple physiological processes of *L. migratoria manilensis*. Especially, *LmUBX*2 might be related to the reproduction of *L. migratoria manilensis*, although further research needs to be performed to confirm this correlation.

Key words: *Locusta migratoria manilensis*; UBX domain-containing protein; *LmUBX*2; gene cloning; expression pattern

1 INTRODUCTION

Ubiquitin regulatory X (UBX) domain was first described in the hypothetical human protein Y33K (Hofmann and Bucher, 1996). Subsequent researches showed that UBX domain-containing proteins are widely distributed in all eukaryotes (Schuberth and Buchberger, 2008). Structural analysis of the UBX domain of FAF-1 and p47 reveal that they are structurally homologous to ubiquitin and act as a p97-binding module (Buchberger *et al.*, 2001; Yuan *et al.*, 2001; Dreveny *et al.*, 2004). The p97, also known as VCP in higher eukaryotes and Cdc48p in yeast, is a homohexameric ATPase (ATPase associated with a variety of activities) involved in multiple cellular processes, including protein degradation through the ubiquitin-proteasome system and homotypic membrane fusion (Woodman, 2003; Wang *et al.*, 2004; Ye, 2006; White and Lauring, 2007).

UBX domain-containing proteins have been identified in a wide range of species (Schuberth and Buchberger, 2008). Based on the sequence homology outside the UBX domain, UBX domain-containing proteins can be divided into several conserved subfamilies, including UBXD-1, UBXD-3, FAF1, p47, SAKS1, TUG, Rep8 and so on (Schuberth and Buchberger, 2008). *Caenorhabditis elegans* possesses six UBX-containing proteins (UBXN-1 to -6), which were dynamically expressed throughout worm's development and in different tissues tested (Yamauchi *et al.*, 2007). All six UBXN proteins are cofactors for CDC-48/p97 and induce embryonic lethal and sterile by the simultaneous knockdown of *ubxn*-1, *ubxn*-2 and *ubxn*-3 (Sasagawa *et al.*, 2010). Caspar, a homologue of human FAF1, was found to be a

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specific negative regulator in the immune deficiency (Imd) signaling pathway of *Drosophila* by inhibiting the Dredd-dependent cleavage of Relish *in vivo* (Kim *et al.*, 2006). In the *caspar* mutant flies, the antimicrobial peptide (AMP) gene *diptericin* was constitutively expressed even in the absence of infections. While, the overexpression of *caspar* was sufficient to suppress the Imd pathway but not the Toll pathway (Kim *et al.*, 2006). The knockdown of *Locusta migratoria FAF*1 (*LmFAF*1) by RNAi caused the hyperactivation of the AMP gene *prolixicin*, suggesting that it shares a similar function as Caspar in *Drosophila* (He *et al.*, 2013a). In major malaria vectors *Anopheles stephensi* and *Anopheles albimanus*, the RNAi-mediated silencing of *caspar* efficiently prevents the development of malaria parasite *Plasmodium falciparum* (Garver *et al.*, 2009).

The oriental migratory locust, *Locusta migratoria manilensis* (Meyen), is an orthopteran pest and a representative member of the hemimetabolous insects for biological studies (Kang *et al.*, 2004; Ma *et al.*, 2006). In previous studies, we identified several UBX domain-containing proteins from *L. migratoria manilensis* including LmFAF1 and LmUBX1, which were classified into the FAF1 subfamily and the UBXD1 subfamily, respectively, based on the sequence characteristics and phylogenetic analysis (He *et al.*, 2013a, 2013b). However, there is no more information about other subfamilies in *L. migratoria manilensis*. In this study, a gene encoding the UBX domain-containing protein of the SAKS1 subfamily was identified through mining the transcriptome database (unpublished data) and designated as *LmUBX*2. Its expression patterns were further investigated at different developmental stages or in different tissues using real-time RT-PCR. Taken together, these results provided an insight into the possible roles of *LmUBX*2 in locust development.

2 MATERIALS AND METHODS

2.1 Experimental insect

Locusta migratoria manilensis (Orthoptera: Acrididae) was originally collected from Hebei Province, China, and reared as previously described (He *et al.*, 2006).

2.2 RNA extraction

Total RNA was extracted from different tissues using Trizol reagent (TaKaRa Dalian, China) according to the manufacturer's protocol and treated with On-Column DNase to remove DNA contamination. RNA concentration and quality were estimated by spectrophotometry and agarose gel electrophoresis, respectively. cDNA was synthesized from 1 μg mRNA using the Smart Race cDNA Amplification kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions.

2.3 Cloning and bioinformatic analysis of *LmUBX*2

The transcriptome database of *L. migratoria manilensis* was analyzed using human SAKS1 (Y33K; LOC51035) as a query. The retrieved sequences were further analyzed by BLASTX against the non-redundant database at the NCBI. The open reading frame (ORF) was predicted by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The theoretical isoelectric point and molecular weight were predicted using ProtParam and Compute pI/Mw (http://web.expasy.org/compute_pi/). The signal peptides were predicted by the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). Motif identification was carried out by ScanProsite (http://prosite.expasy.org/) (De Castro *et al.*, 2006). Sequence similarity was analyzed by BLAST searching in GenBank (http://www.ncbi.nlm.nih.gov/). Multi-sequence alignment was generated using Clustal X (Thompson *et al.*, 1997). Homology modeling was performed by the SWISS-MODEL server (http://swissmodel.expasy.org/) (Biasini *et al.*, 2014). An unrooted phylogenetic tree was constructed by MEGA 4.0 (Tamura *et al.*, 2007) using the neighbor-joining method with 1 000 bootstrap replications.

2.4 Expression patterns of *LmUBX*2 at different developmental stages and in different tissues

Gene expression was analyzed at different developmental stages including embryo (mixed with various developmental stages), 1st, 2nd, 3rd, 4th and 5th instar nymphs, and adult. Locusts were collected in whole bodies (remains in the alimentary canal were removed) at 2 d after moulting, and 5 to 10 individuals were collected for each sample. Meanwhile, gene expression was also examined in the midgut, ovary, testis, integument and fat body. Ovary and testis were dissected from 2 day-old female and male adults. Other tissues were collected from female adults. The locusts were dissected on ice, and the tissues were isolated and stored at −80℃.

Real-time PCR was carried out using SYBR PrimeScript RT-PCR Kit (TaKaRa, Dalian, China) and CFX Connect™ (Bio-Rad). First-strand cDNA was synthesized from 1 μg of total RNA using random hexamers. Gene specific primers were listed in Table 1. The PCR conditions were as follows: 95℃ for 15 s; 40 cycles of 95℃ for 10 s, 58℃15 s,

72℃ 20 s; and then 95℃ for 15 s and 60℃ for 30 s. The *Armadillo* was simultaneously quantified as an internal control. The relative expression level was measured using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Each sample had three replicates and the experiment was repeated three times.

Table 1　Primers used in this study

Primer name	Sequence (5′-3′)	Purpose
UBX2-F	CTGCCTCAACTATGCCTACAC	*LmUBX2* real-time PCR
UBX2-R	GCCTAACTGCTGCCAACTG	*LmUBX2* real-time PCR
18SF	TTAAGCCATGCATGTCTAAGTA	18S rRNA real-time PCR
18SR	TCTCAGGCTCCCTCTCCGGAATCG	18S rRNA real-time PCR

3　RESULTS

3.1　Cloning and characterization of *LmUBX2*

A gene fragment (Unigene38793) was retrieved and showed 40% - 64% identity to other UBX domain-containing proteins at the amino acid level. This gene was named as *LmUBX2* and was deposited into GenBank (accession no. KP280173). *LmUBX2* contains a 1 020-bp ORF encoding a 339-aa protein with a theoretical MW of 37.80 kDa and a pI of 6.03. The genomic sequences of *LmUBX2* were identified by BLAST against the *L. migratoria* whole genome shotgun (WGS) sequences (Wang *et al.*, 2014). The entire ORF of *LmUBX2* is distributed in 6 contigs and consists of 7 exons (Fig. 1: A). Except the intron 1, the length of other 5 introns remains unknown due to the discontinuous genomic sequences (Fig. 1: A).

Secondary structural prediction showed that LmUBX2 possess 51.33% α-helix, 5.90% extended strand, 3.54% β-turn and 39.23% random coil. Therefore, α-helix and random coil represent the dominant secondary structures. Domain analysis revealed that LmUBX2 has multiple functional domains including an N-terminal ubiquitin-associated (UBA) domain (aa 2 - 40), and a C-terminal UBX domain (aa 253 - 336), which respectively exhibited 56% - 79% and 51% - 83% sequence identities to other UBA and UBX domains (Fig. 1: B). The modeling structures of UBA and UBX domains of LmUBX2 and human SAKS1 were constructed through on-line SWISS-MODEL service (Fig. 1: E, F, G and H). Comparative analysis showed that the structures of UBA and UBX domains are highly conserved (Fig. 1), which are also consistent with the conserved amino acid sequences of UBA and UBX domain (Fig. 1: C, D).

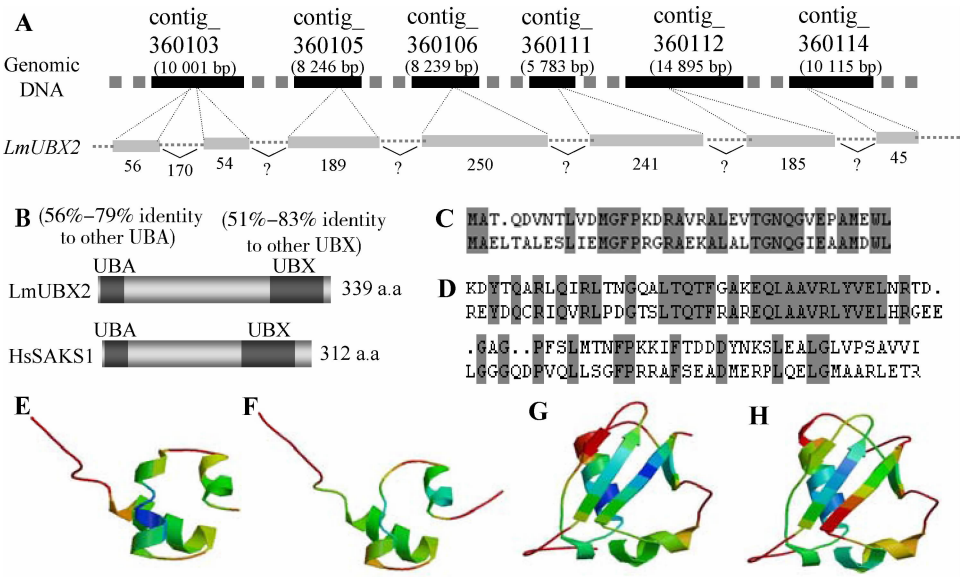

Fig. 1　Genomic structure and domains analysis of *LmUBX2* of *Locusta migratoria manilensis*

A: Genomic structure of *LmUBX2*. The bold dotted lines represent the gaps between the contigs, while the thin dotted lines represent introns, or the 5-terminal and 3-terminal untranslated regions. "?" indicates that the length of the intron remains unknown. The figures above the bold black line represent the length of the contigs, while the figures below the bold grey line represent the length of the exons. B: Domains of *LmUBX2*. C: Alignment of UBA domain of LmUBX2 and human SAKS1. D: Alignment of UBX domain of LmUBX2 and human SAKS1. Identical residues (>50%) are shaded with dark gray. The comparison of the modeling structures of UBA domain LmUBX2 (E) and SAKS1 (F). The comparison of the modeling structures of UBX domain of LmUBX2 (G) and SAKS1 (H). LmUBX2: *Locusta migratoria manilensis*, KP280173; HsSAKS1: *Homo sapiens*, LOC51035; UBX: Ubiquitin regulatory X domain; UBA: Ubiquitin-associated domain.

BLAST analysis showed that LmUBX2 is a relatively conserved protein sharing 37% –64% amino acid sequence identities with other UBX domain-containing proteins from plants, worms, insects, mammals and fungi. Amongst them, LmUBX2 is most closely related to the homologue (GenBank accession no. XP_005814923) from *Xiphophorus maculatus* with 64% amino acid sequence identity. Phylogenetic analysis of the 17 UBX domain-containing proteins, based on amino acid sequences, is presented in Fig. 2. The tree topology indicated that LmUBX2 was clustered with other SAKS1 homologues, suggesting that LmUBX2 is a member of the SAKS1 subfamily. While LmUBX1 andLmFAF1 were grouped into the UBXD1 subfamily and the FAF1 subfamily, respectively (Fig. 2).

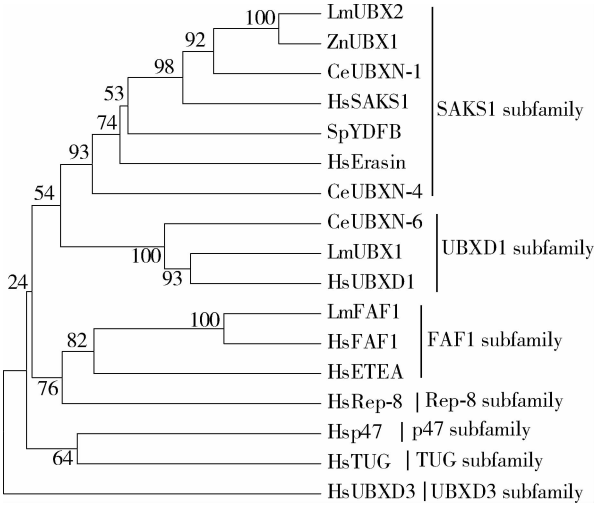

Fig. 2 Phylogenetic tree of LmUBX2 of *Locusta migratoria manilensis* and homologues of other species constructed by MEGA 4 with the neighbor-joining method based on amino acid sequences

The number at each node indicates the percentage of bootstrapping after 1 000 replications. LmUBX2: *Locusta migratoria manilensis* UBX2, KP280173; ZnUBX1: *Zootermopsis nevadensis* UBX1, KDR19499; CeUBXN1: *Caenorhabditis elegans* UBXN-1, CCD69888; HsSAKS1: *Homo sapiens* SAKS1, LOC51035; SpYDFB: *Schizosaccharomyces pombe* YDFB, SPAC17C9.11c; erasin: *H. sapiens*, ABM90426; CeUBXN4: *C. elegans* UBXN-4, NP_498856; CeUBXN6: *C. elegans* UBXN-6, NP_500648.2; LmUBX1: *L. migratoria manilensis* UBX1, JN661173; HsUBXD1: *H. sapiens* UBXD1, AF272893; LmFAF1: *Locusta migratoria manilensis* FAF1, AB758246; HsFAF1: *H. sapiens* FAF1, Q9UNN5; HsETEA: *H. sapiens*, AAH14001; HsRep-8: *H. sapiens* Rep-8, D83767; Hsp47: *H. sapiens* p47, BC002801; HsTUG: *H. sapiens* ASPL, AF324219.1; HsUBXD3: *H. sapiens* UBXD3, P_689589.1.

3.2 Expression of *LmUBX2* at different developmental stages

LmUBX2 expression pattern was analyzed throughout the life cycle by real-time PCR. The highest expression level was found at adult stage ($F = 48.1560$, $P = 0.0001$), while the transcript levels did not significantly vary during the embryo and nymphal development ($F = 2.0630$, $P = 0.1412$) (Fig. 3: A).

3.3 Expression of *LmUBX2* in different tissues

The transcript levels of *LmUBX2* significantly varied in different tissues ($F = 554.435$, $P = 0.0001$) (Fig. 3: B). It was highly expressed in ovary, followed by testes. Besides, the difference between ovary and testes ($t = 1.6949$, $P = 0.1653$) was significant ($t = 5.8330$, $P = 0.0043$). The transcript level in the ovaries and testes were 6.77-, and 5.37-fold higher than that in the integument, respectively.

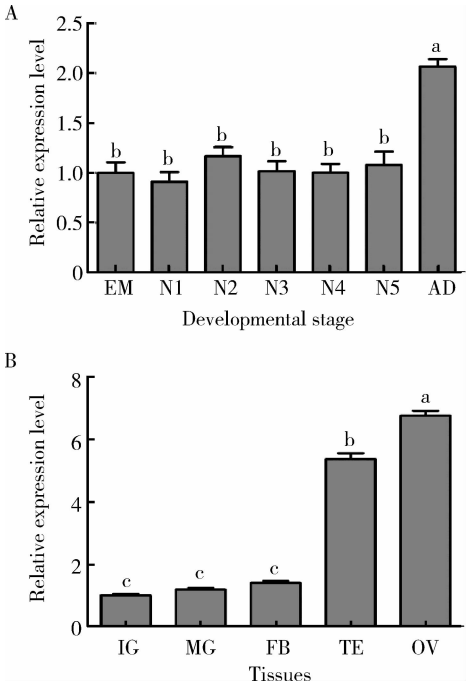

Fig. 3 Gene expression profiles of *LmUBX2* throughout the life cycle (A) and in different tissues (B) of *Locusta migratoria manilensis*

Armadillo was used as an endogenous control. EM: Embryo; N1: 1st instar nymph; N2: 2nd instar nymph; N3: 3rd instar nymph; N4: 4th instar nymph; N5: 5th instar nymph; AD: Adult; MG: Midgut; OV: Ovary; TE: Testis; IG: Integument; FB: Fatty body. Columns topped by different letters indicate significantly different means at the 0.01 level by ANOVA analysis.

4 DISCUSSION

In this study, *LmUBX2* was identified and characterized from *L. migratoria manilensis* transcriptome dataset. Phylogenetic analysis indicated that LmUBX2 was clustered with other SAKS1 proteins, suggesting that LmUBX2 could be a member of the SAKS1 subfamily (Fig. 2). Previous studies revealed that *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, *Homo sapiens*, *C. elegans* genome contains 15, 7, 13 and 10 UBX domain-containing protein genes, respectively (Rancour *et al.*, 2004; Schuberth *et al.*, 2004; Yamauchi *et al.*, 2007; Schuberth and Buchberger, 2008).

Therefore, the three UBX domain-containing proteins, LmUBX1, LmUBX2 and LmFAF1, obtained from the transcriptome data might not represent the entire repertoire of the UBX domain-containing protein family of *L. migratoria manilensis*. Additionally, the UBX domain-containing protein genes of *L. migratoria manilensis* remain to be discovered. Fortunately, the *L. migratoria manilensis* genome has been sequenced and released (Wang *et al.*, 2014), which will facilitate the genome-wide characterization of all UBX domain-containing proteins.

Expression analysis results showed that *LmUBX*2 was expressed throughout the developmental process of *L. migratoria manilensis* (Fig. 3), suggesting that it might be involved in multiple physiological processes of *L. migratoria manilensis*. *LmUBX*1 and *LmFAF*1 were also distinctly expressed at each developmental stage (He *et al.*, 2013a, 2013b). Of particular concern is that *LmUBX*2 was highly expressed in ovary and testis (Fig. 3: B). Similarly, *LmUBX*1 was mainly expressed in ovary and testis (He *et al.*, 2013b), and *LmFAF*1 was simultaneously highly expressed in fat body, testis and ovary (He *et al.*, 2013a). Taken together, our results suggested that these three UBX genes *LmUBX*1, *LmUBX*2 and *LmFAF*1, are regulated in strikingly different manners.

The function of UBX proteins of the SAKA1 subfamily remains unknown. The *C. elegans UBXN*-1, the same subfamily with *LmUBX*2, was expressed ubiquitously through all developmental stages (Yamauchi *et al.*, 2007). Knockdown of *ubxn*-1, *ubxn*-2 and *ubxn*-3 induced embryonic lethal and sterile phenotypes (Sasagawa *et al.*, 2010). Furthermore, TRA-1A, a key factor in the sex determination pathway, accumulated in worms and inhibited spermatogenesis when *ubxn*-1, *ubxn*-2 and *ubxn*-3 were simultaneously knocked down. This suggests that UBXN-1, UBXN-2 and UBXN-3 may control spermatogenesis via the degradation of TRA-1A (Sasagawa *et al.*, 2010). The higher abundance of *LmUBX*2 in ovary and testis indicates that *LmUBX*2 might be involved in the reproduction of *L. migratoria manilensis*. But further research is required to confirm these correlations.

UBX domain-containing proteins function as cofactors of p97/CDC48, which is involved in multiple ubiquitin-related processes including protein degradation through the ubiquitin-proteasome system. LmUBX2, like *C. elegans* UBXN-1 and human SAKA1, contains multi-ubiquitin-related domains, including an N-terminal UBA and a C-terminal UBX domain (Fig. 1: B). UBX domain is structurally homologous to ubiquitin and acts as a p97-binding module, whereas the UBA domain seems to be responsible for recruiting and stabilizing ubiquitinated proteasomal substrates (Schuberth and Buchberger, 2008). Existing results reveal that UBXN-1, UBXN-2 and UBXN-3 are likely involved in the degradation of TRA-1A (Sasagawa *et al.*, 2010). Moreover, ovary and testis cells are renewed at a relatively faster rate accompanying with exuberant protein metabolization. Therefore, we speculated that LmUBX2 may be involved in the degradation of some proteins in ovary and testis of *L. migratoria manilensis*, but further studies need to be performed to test this hypothesis.

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东亚飞蝗 UBX 结构域包含蛋白基因 *LmUBX2* 的克隆和表达分析

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摘要: 【目的】UBX 结构域包含蛋白是 p97/CDC48 的辅助因子。p97 在泛素化相关的多种细胞过程中起着重要的作用,如依赖泛素-蛋白酶体系统的蛋白质降解和同型膜融合等。本研究旨在克隆东亚飞蝗 *Locusta migratoria manilensis* (Meyen)的 UBX 结构域包含蛋白基因,分析其组织和发育表达格局,为进一步研究 UBX 结构域包含蛋白基因的功能奠定基础。【方法】通过分析东亚飞蝗的转录组数据克隆 UBX 结构域包含蛋白基因,采用实时定量 PCR 技术分析该基因在不同发育时期和成虫不同组织中的表达水平。【结果】克隆到东亚飞蝗的一个 UBX 结构域包含蛋白基因,命名为 *LmUBX2*。*LmUBX2* 开放阅读框长 1 020 bp,编码 399 个氨基酸,预测分子量和等电点分别为 37.8 kDa 和 6.03,与其他 UBX 结构域包含蛋白的氨基酸一致性为 37% ~64%,N 端和 C 端分别有一个保守的 UBA 结构域和 UBX 结构域。序列比较和系统发育分析发现 *LmUBX2* 属于 SAKS1 亚家族。定量分析发现,*LmUBX2* 在整个生命周期中都有表达,但成虫期的表达水平最高;在检测的所有组织中都有表达,但在精巢和卵巢中表达水平最高。【结论】研究结果说明 *LmUBX2* 可能参与东亚飞蝗多种生理过程,尤其可能与东亚飞蝗的生殖有关,但还需深入研究。

关键词: 东亚飞蝗; UBX 结构域包含蛋白; *LmUBX2*; 基因克隆; 表达模式

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